VERIFICATION OF TRANSLATION

Re : Japanese Patent Application No. 11-234262

I, Sonoko TSUKIYAMA, of c/o Hosoda International Patent Office, OMM Building 5th Floor, P.O. Box 26, 1-7-31 Otemae, Chuo-ku, Osaka 540-6591, JAPAN, hereby declare that I am the translator of the documents attached and certify that the following is a true translation of the best of my knowledge and belief.

Dated this 29th day of October, 2004

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Applicant(s): Takara Shuzo Co., Ltd.

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[List of Annexed Documents]

[Document]	Specification	1
[Document]	Drawings	1
[Document]	Abstract	1

[Requirement of Proof] Required

[Document] Specification

[Title of the Invention] Therapeutic Agents

[Claims]

[Claim 1] A therapeutic agent or prophylactic agent for a disease requiring regulation of cytokine production, a disease requiring immunopotentiation, or a disease requiring nitrogen monoxide production, characterized in that the therapeutic agent or prophylactic agent comprises as an effective ingredient a fucoidan and/or a degradation product thereof.

[Claim 2] The therapeutic agent or prophylactic agent according to claim 1, wherein the fucoidan is derived from an algae or Echinodermata.

[Claim 3] The therapeutic agent or prophylactic agent according to claim 1 or 2, wherein the cytokine is an interleukin or an interferon.

[Claim 4] The therapeutic agent or prophylactic agent according to any one of claims 1 to 3, wherein the interferon is interferon-γ.

[Claim 5] The therapeutic agent or prophylactic agent according to any one of claims 1 to 3, wherein the interleukin is interleukin-12.

[Claim 6] A food or beverage for regulation of cytokine production, a food or beverage for immunopotentiation, or a food or beverage for induction of nitrogen monoxide production, wherein a fucoidan or a degradation product thereof is contained in, added to, and/or diluted in the food or beverage.

[Claim 7] The food or beverage according to claim 6, wherein the fucoidan is derived from an algae or Echinodermata.

[Claim 8] The food or beverage according to claim 6 or 7, wherein the cytokine is an interleukin or an interferon.

[Detailed Description of the Invention]

[0001]

[Technical Field to Which the Invention Pertains]

The present invention relates to applications of physiologically active substances derived from aquatic organisms as medicaments, foods or beverages.

[0002]

[Prior Art]

As physiologically active substances derived from aquatic organisms, a fucoidan has been known. This fucoidan is a sulfated fucose-containing polysaccharide contained in algae, Echinodermata, or the like. Namely, the fucoidan comprises a sulfated fucose as a constituting saccharide.

[0003]

As physiological action of the fucoidan, cancer proliferation-suppressing activity, cancer metastasis-suppressing activity, anticoagulation activity, antiviral activity and the like have been known, and the developments of applications as medicaments have been expected of the fucoidan.

[0004]

[Problems to Be Solved by the Invention]

The present invention is based on the findings of new physiological action for the fucoidan, and its object is to provide a medicament, food or beverage, utilizing the regulatory action for cytokine production or the like of the fucoidan.

[0005]

[Means to Solve the Problems]

Summarizing the present invention, a first invention of the present invention relates to a therapeutic agent or prophylactic agent for a disease requiring regulation of cytokine production, a disease requiring

immunopotentiation or a disease requiring nitrogen monoxide production, characterized in that the therapeutic agent or prophylactic agent comprises as an effective ingredient a fucoidan and/or a degradation product thereof.

A second invention of the present invention relates to a food or beverage for regulation of cytokine production, a food or beverage for immunopotentiation, or a food or beverage for induction of nitrogen monoxide production, wherein a fucoidan or a degradation product thereof is contained in, added to, and/or diluted in the food or beverage.

The fucoidan used in the first invention and the second invention of the present invention is not particularly limited. There are preferably exemplified a fucoidan derived from an algae, or Echinodermata.

The term "cytokine" is the cytokine on which the fucoidan or a degradation product thereof can exhibit an action for regulation of production. The cytokine includes, for instance, an interleukin such as interleukin-12; and an interferon such as interferon-γ.

Further, the term "immunopotentiating action" refers to action for activating immunization ability of a living body by the fucoidan or a degradation product thereof, which is exemplified by enhancing action for cytotoxic T cell.

[0006]

[Modes for Carrying out the Invention]

The fucoidan used in the present invention refers to a substance in which a sulfated fucose is contained as a constituent. The fucoidan is not particularly limited, as long as the fucoidan has action for regulation of cytokine production, for instance, action for regulation of interferon-γ production, or action for regulation of interleukin-12 production during antigen presentation reaction

between antigen presenting cells (APCs) and T cells; action for immunopotentiation, for instance, enhancing action for cytotoxic T cells, action for induction of nitrogen monoxide production. For instance, especially marine algae such as Laminariales, Chordariales and Fucales, including *Kjellmaniella crassifolia*, *Kjellmaniella gyrata*, *Fucus*, *Cladosiphon okamuranus*, *Undaria*, *Undaria pinnatifida*, *Ecklonia kurome*, *Eisenia bicyclis*, *Ecklonia cava*, Giant kelp, *Lessonia nigrescence* and *Ascophyllum nodosum* richly contain fucoidans suitable for the use in the present invention. This is why they are preferable as the raw material. In addition, there may be used fucoidans derived from Echinodermata, for instance, sea cucumber, Echnoidea, Asterozoa, and the like.

[0007]

These fucoidans may be prepared each by a known method. There can be used in the present invention a purified product of a fucoidan, the fucoidan or the like.

[0008]

For instance, a fucoidan is prepared from *Kjellmaniella crassifolia*, and the resulting fucoidan can be further separated into glucuronic acid-containing fucoidan (referred to as "U-fucoidan") and glucuronic acid non-containing fucoidan (referred to as "F-fucoidan"). Each of these fucoidans can be used as an effective ingredient of the present invention.

[0009]

After the preparation of the fucoidans from *Kjellmaniella crassifolia*, U-fucoidan and F-fucoidan are separated by using an anionic exchange resin, a surfactant or the like. The existing ratio of U-fucoidan to F-fucoidan derived from *Kjellmaniella crassifolia* is about 1:2. U-fucoidan contains fucose,

mannose, galactose, glucuronic acid and the like, and its sulfate content is about 20%. F-fucoidan contains fucose and galactose, and its sulfate content is about 50%. The molecular weight for both substances is distributed, centering about 200000 (*Summary of 18th Sugar Symposium*, p. 159, 1996).

[0010]

U-fucoidan and F-fucoidan can be separated, for instance, by applying a fucoidan solution prepared from *Kjellmaniella crassifolia* onto DEAE-Cellulofine A-800 column, and carrying out elution by the concentration gradient technique using NaCl-containing buffer. One of the examples is shown in Figure 1. Concretely, Figure 1 is a diagram showing the separation of U-fucoidan and F-fucoidan, wherein the former peak in the figure is U-fucoidan, and the latter peak is F-fucoidan.

[0011]

Also, for instance, each of the fucoidan derived from *Fucus*, the fucoidan derived from *Cladosiphon okamuranus*, the fucoidan derived from *Undaria*, and the fucoidan derived from *Undaria pinnatifida* can be prepared by a known method and used in the present invention.

The sea cucumber containing the fucoidan includes, for instance, sea cucumbers described in Japanese Patent Laid-Open No. Hei 4-91027. The fucoidan can be prepared from sea cucumbers in accordance with the method described in the publication.

[0012]

In addition, the degradation product of the fucoidan having action for regulation of cytokine production, immunopotentiation, and induction of nitrogen monoxide production used in the present invention can be prepared by a

known method such as an enzymological method, a chemical method, or a physical method, and a desired degradation product having the desired inducing action for growth factor production can be selected and used.

The method for preparing the degradation product of the fucoidan used in the present invention includes, for instance, an acid degradation method. By subjecting the fucoidan to an acid degradation, there can be prepared a degradation product having action for regulation of cytokine production, immunopotentiation, or induction of nitrogen monoxide production.

[0013]

The conditions for the acid degradation of the fucoidan used in the present invention are not particularly limited, as long as the conditions enable to generate the degradation product having action for regulation of cytokine production, immunopotentiation, or induction of nitrogen monoxide production (hereinafter referred to as "degradation product of the present invention").

For instance, the fucoidan is dissolved or suspended in an acid and subjected to the reaction, thereby generating a degradation product of the present invention. Also, the reaction mixture may be heated during the reaction, thereby shortening the time period required for the generation of the degradation product of the present invention.

The kinds of the acids for dissolving or suspending the fucoidan are not particularly limited. There can be used an inorganic salt of hydrochloric acid, sulfuric acid, nitric acid or the like; an organic acid such as citric acid, formic acid, acetic acid, lactic acid or ascorbic acid; and a solid acid such as cationic exchange resin, cationic exchange fiber or cationic exchange membrane.

[0014]

The concentration of the acid is not particularly limited, and the acid can be used at a concentration of from 0.0001 to 5 N, preferably from 0.01 to 1 N or so. In addition, the reaction temperature is not particularly limited, and the reaction temperature may be set at from 0° to 200°C, preferably from 20° to 130°C.

In addition, the reaction time is not particularly limited, and the reaction time may be set at from several seconds to several days. The kinds and the concentration of the acids, the reaction temperature, and the reaction time may be properly selected depending upon the generated amount of the degradation product of the present invention and the degree of polymerization of the degradation product. For instance, during the manufacture of the degradation product of the present invention, an organic acid such as citric acid, lactic acid or malic acid is used, the concentration of the acid is properly selected from the range of several dozens mM to several M, the heating temperature is properly selected from the range of 50° to 110°C, preferably 70° to 95°C, and the heating time is properly selected from the range of several minutes to 24 hours, whereby the degradation product of the present invention can be prepared. The acid degradation product of the fucoidan is exemplified by the acid degradation product of the fucoidan derived from *Kjellmaniella crassifolia*, and this degradation product can be used as dietary fiber having new physiological functions of strong action for regulation of cytokine production, especially regulation of interferon-γ production during antigen presentation reaction between APCs and T cells; immunopotentiation, especially enhancing action for cytotoxic T cells; and induction of nitrogen monoxide production.

[0015]

The degradation product of the present invention can be fractionated on the basis of its action for regulation of cytokine production, immunopotentiation, or induction of nitrogen monoxide production. For instance, the acid degradation product can be further fractionated based on a molecular weight by means of a gel filtration method, a fractionation method using a molecular weight fractionation membrane, or the like.

As an example of gel filtration method, Cellulofine GCL-300 can be used to prepare any molecular weight fractions, for instance, one having a molecular weight exceeding 25000, one having a molecular weight of 25000 to exceeding 10000, one having a molecular weight of 10000 to exceeding 5000, one having a molecular weight of 5000 or less. Cellulofine GCL-25 can be used to prepare any molecular weight fractions from the fraction having a molecular weight of 5000 or less, for instance, one having a molecular weight of 5000 to exceeding 3000, one having a molecular weight of 3000 to exceeding 2000, one having a molecular weight of 2000 to exceeding 1000, one having a molecular weight of 1000 to exceeding 500, one having a molecular weight of 500 or less.

[0016]

In addition, the molecular weight fractionation can be industrially carried out by using an ultrafiltration membrane. For instance, a fraction having a molecular weight of 30000 or less can be prepared by using FE10-FUSO382 manufactured by DAICEL CHEMICAL INDUSTRIES, LTD., or a fraction having a molecular weight of 6000 or less can be prepared by using FE-FUS-T653 manufactured by the same. Further, a fraction having a molecular weight of 500 or less can be obtained by using a nanofilter membrane. Any molecular weight fractions can be prepared by combining these gel filtration

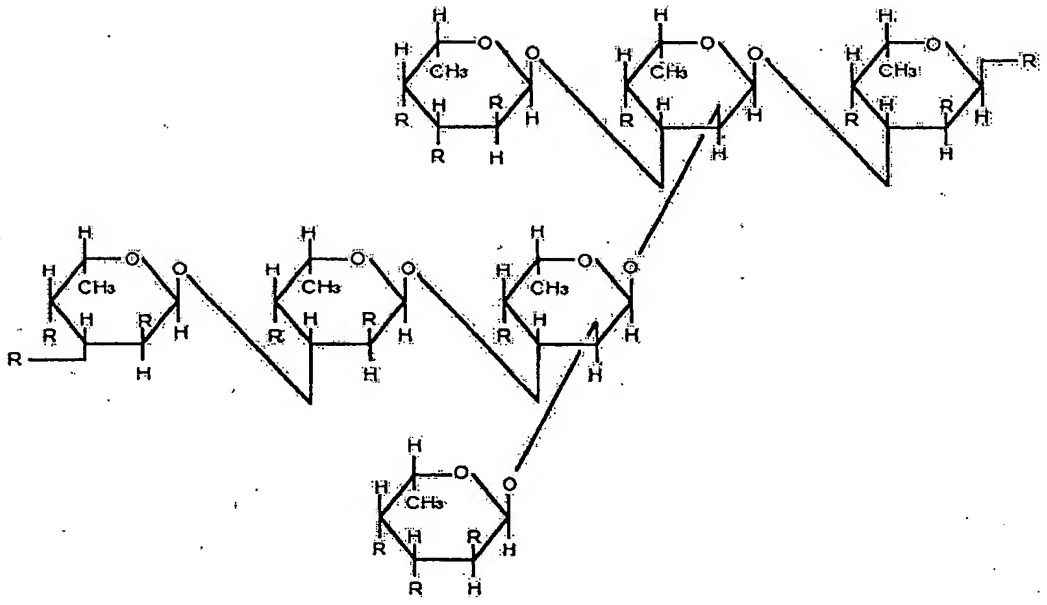
methods and molecular weight fractionation methods.

[0017]

The degradation product of the fucoidan having action for regulation of cytokine production, immunopotentiation, or induction of nitrogen monoxide which can be used in the present invention is exemplified by the compounds represented by the formulas (Ka 1) to (Ka 3), and these compounds can be prepared in accordance with the methods disclosed in WO 97/26896, and the specification for International Application No. PCT/JP99/00606. A fucoidan and an oligosaccharide having a repeating structure of the compound represented by the formula (Ka 1) can be also favorably used in the present invention.

[0018]

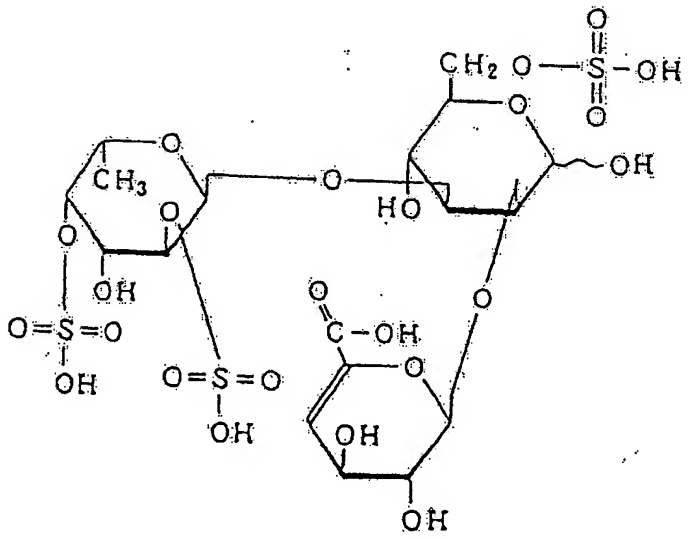
[Ka 1]



wherein R is OH or OSO_3H;

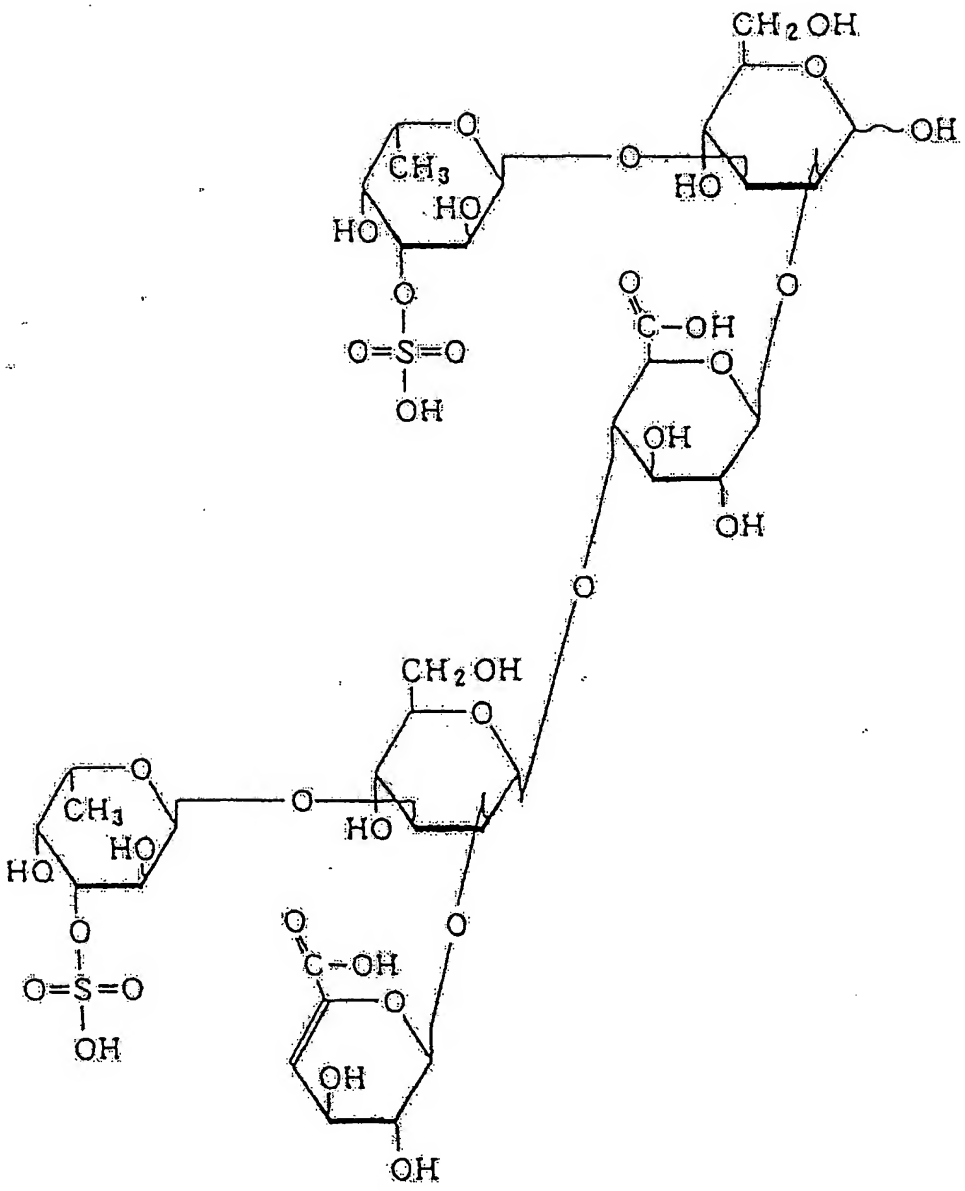
[0019]

[Ka 2]



[0020]

[Ka 3]



[0021]

Examples of the compound represented by the formula (Ka 1) include the compound represented by the formula (Ka 4) given later.

[0022]

The cytokine requiring production regulation in the present invention is not particularly limited. There are exemplified, for instance, interleukins (IL)-1 to -13, interferon (IFN)-α, IFN-β, IFN-γ, lymphotoxins, tumor necrosis factors (TNFs), stem cell factors (SCFs), granulocyte macrophage-colony stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage-colony stimulating factor (M-CSF), and the like.

[0023]

These cytokines include those involved in expression and regulation of various cellular immune responses such as delayed-type hypersensitive reactions and target cytotoxicity (IL-2, IFN-γ, TNF-α, TNF-β and the like); those involved in the regulatory functions in the antibody production mechanism (IL-2, IL-4, IL-5, IL-6, and the like); those directly exhibiting suppressive action for proliferation or destroying action on tumor cells (TNF-α, TNF-β, IFN and the like); those capable of accelerating proliferation and differentiation of hematopoietic stem cells and precursor cells in the bone marrow (IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, GM-CSF, G-CSF, M-CSF, and the like); those involved in inflammatory reactions (IL-1, IL-6, IL-8, TNF-α, TNF-β, IFN and the like); those involved in allergic reaction (IL-3, IL-4, IL-5 and the like); those capable of enhancing activity of NK cells (IL-12); and the like. By regulating the production of these cytokines, there can be treated or prevented a disease requiring expression and regulation of cellular immune response such as cancer; a disease requiring regulation of an antibody production such as autoimmune disease; a disease requiring differentiation of cells; or a disease requiring suppression of inflammation.

[0024]

The fucoidan and a degradation product thereof used in the present invention have an ability for regulation of cytokine production, so that a therapeutic agent or prophylactic agent for the above-mentioned disease requiring cytokine production can be prepared by using these compounds as effective ingredients.

The fucoidan and a degradation product thereof used in the present invention have immunopotentiating action such as enhancing action for cytotoxic T cells, so that a therapeutic agent or prophylactic agent for a disease requiring enhancement of cytotoxic T cells, such as viral diseases and cancer diseases can be prepared by using these compounds as effective ingredients.

The fucoidan and a degradation product thereof used in the present invention have action for induction of nitrogen monoxide production, so that a therapeutic agent or prophylactic agent for a disease requiring nitrogen monoxide production, such as arteriosclerosis requiring flaccidity of vascular smooth muscle, suppression of adhesion of thrombocytes, granulocytes and monocytes to vascular walls, inhibition of proliferation of secretory smooth muscle cells, and the like can be prepared by using these compounds as effective ingredients.

[0025]

The therapeutic agent or prophylactic agent of the present invention can be obtained by combining the fucoidan and/or a degradation product thereof as an effective ingredient with a known medicinal vehicle to make a preparation. The preparation is generally produced by formulating the fucoidan and/or a degradation product thereof with a pharmaceutically acceptable liquid or solid vehicle, and optionally adding a solvent, a dispersant, an emulsifier, a buffer, a

stabilizer, an excipient, a binder, a disintegrant, a lubricant, or the like, and forming the resulting mixture into a solid agent such as a tablet, a granule, a powder, a fine powder, or a capsule, or a liquid agent such as a general liquid agent, a suspension agent, or an emulsion agent. In addition, a dry product which can be made into a liquid form by adding an appropriate vehicle before use can be also prepared.

[0026]

The pharmaceutical vehicle can be properly selected depending upon the administration embodiment and the preparation form of the therapeutic agent or prophylactic agent of the present invention. In the case of an orally administered preparation, for instance, starch, lactose, saccharose, mannitol, carboxymethyl cellulose, cornstarch, inorganic salts and the like are available. In addition, during the preparation of the orally administered preparation, a binder, a disintegrant, a surfactant, a lubricant, a fluidity accelerator, a flavor, a colorant, a perfume, or the like can be further formulated.

[0027]

On the other hand, in the case of a non-orally administered preparation, according to the conventional method, the preparation can be produced by dissolving or suspending the fucoidan and/or a degradation product thereof, which is an effective ingredient of the present invention, in distilled water for injection, physiological saline, aqueous glucose solution, vegetable oil for injection, sesame oil, peanut oil, soybean oil, corn oil, propylene glycol, polyethylene glycol or the like as a diluent, and optionally adding a sterilizer, a stabilizer, an osmotic regulator, a soothing agent, or the like.

[0028]

The therapeutic agent or prophylactic agent of the present invention may be administered via an administration route appropriate for each of the preparation form. The administration method is not limited to specific one. The agent can be administered internally or externally (or topically) or by injection. The injection can be administered, for instance, intravenously, intramuscularly, subcutaneously, intracutaneously, or the like. External preparations include a suppository.

[0029]

The dose for the therapeutic agent or prophylactic agent of the present invention is changeable and properly set depending upon its preparation form, administration method, purpose of use, age, body weight, symptom or the like of the patient to which the agent is applied, or the like. The dose for adult per day is generally from 0.1 to 2000 mg/kg as the amount of the fucoidan and/or a degradation product thereof contained in the preparation. As a matter of course, the dose varies depending upon various conditions, so that an amount smaller than the dose mentioned above may be sufficient, or an amount exceeding the dose range may be required. The agent of the present invention can be directly orally administered, or the agent can be added to any foodstuffs to take it on a daily basis. Also, the fucoidan and/or a degradation product thereof may be used as a raw material of foodstuffs for regulation of cytokine production, foodstuffs for immunopotentiation, or foodstuffs for induction of nitrogen monoxide production.

[0030]

In addition, according to the present invention, there is provided an agent for regulation of IFN-γ, an agent for regulation of IL-12, an enhancing agent for

cytotoxic T cells, an agent for induction of nitrogen monoxide production, each comprising as an effective ingredient the fucoidan and/or a degradation product thereof. These agents can be prepared as the above-mentioned therapeutic agent or prophylactic agent, and can be applied to the diseases requiring the administration of the agent.

[0031]

As the induction of IFN-γ production from lymphocytes, there have been known induction via direct stimulation of T cells by mitogen such as Con A, and via intercellular interaction between antigen presenting cells (APCs) and T cells. The former is the induction by direct binding of the mitogen to T cell receptor (TCR), and the latter is stimulation of TCR by APCs and the induction by IL-12 produced from APC, and the like.

Conventionally, there has been reported that a fucoidan derived from *Laminaria japonica* induces IFN-γ production from spleen lymphocytes derived from normal mouse to enhance the activation of NK cells (*Chugoku Kaiyo Yakubutsu Zasshi* (Zhongguo Haiyang Yaown), 1995, Third Period, 9-13). However, this is the induction caused by direct stimulation to T cells.

On the other hand, regarding the action for induction of IFN-γ presently confirmed by the present inventors, as shown in Examples, the induction action of IFN-γ of the fucoidan and a degradation product thereof was not found in the direct stimulation or the alloantigen stimulation of lymphocytes, and the IFN-γ production was increased during the antigen presentation reaction between APC and T cell.

From the above fact, there can be deduced that the fucoidan and a degradation product thereof used in the present invention act on APCs upon

antigen presentation to enhance IL-12 production, thereby indirectly enhancing INF-γ production from T cells.

In fact, there has been also found the induction of IL-12 in culture supernatant in which IFN-γ is induced by the fucoidan and a fraction thereof, and the enhancement of IFN-γ production via IL-12 during the antigen presentation between APC and T cells is thought to be the functional mechanism of IFN-γ induction by the fucoidan and a fraction thereof. The fucoidan described in the above report which studies the direct induction action is different from the fucoidan used in the present invention, so that its results show completely different actions.

[0032]

In addition, it was also shown in a system using a macrophage cell line, one of antigen presenting cells, that the fucoidan and a degradation product thereof induces nitrogen monoxide production, and exhibits the immunopotentiating effect through the induction. However, even in this system, there was no induction of IFN-γ production by the fucoidan and a degradation product thereof. It is considered that the fucoidan and a degradation product thereof used in the present invention does not have activity for direct induction of IFN-γ production, and since nitrogen monoxide production seems to be enhanced parallel to IL-12 production, the enhancement of IFN-γ production by the fucoidan and a degradation product thereof in the present invention is caused by IL-12 production from the stimulation of APC during antigen presentation, not by the induction by direct stimulation of T-cells. From this aspect, the fucoidan and a degradation product thereof used in the present invention exhibit completely different action from that of the above-mentioned report which

studies on the direct induction action.

Here, IFN-γ is produced when a T cell receptor of T cell (Th1) is stimulated with antigen presenting cell (APC) and the like, and the IFN-γ production is enhanced by IL-12 produced from APC. IFN-γ activates cellular immunity involving cytotoxic T cell, NK cell, macrophage, and the like in a case such as viral infection, mycosis, and cancer diseases, thereby acting to enhance biophylaxis ability.

On the other hand, IFN-γ has been considered to be effective for the treatment of asthma, pollinosis, atopic dermatitis, and the like by suppressing the activation of Th2 which is causative of incidence in allergic diseases.

[0033]

Next, since the food or beverage which is prepared by containing, adding and/or diluting the fucoidan and/or a degradation product thereof having action for regulation of cytokine production, immunopotentiation, and induction of nitrogen monoxide production has action for regulation of cytokine production, immunopotentiation, or induction of nitrogen monoxide production, the food or beverage is very useful in amelioration or prevention of symptoms for a disease requiring regulation for cytokine production, a disease requiring immunopotentiation, or a disease requiring nitrogen monoxide production, which is sensitive to the fucoidan or a degradation product thereof.

[0034]

The method for manufacturing the food or beverage of the present invention is not particularly limited. For instance, the method includes, cooking, processing, and any methods for manufacturing food or beverage generally employed, as long as the fucoidan and/or a degradation product thereof as an

effective ingredient is contained in, added to, and/or diluted with the resulting food or beverage, wherein the fucoidan and or a degradation product thereof has action for regulation of cytokine production, immunopotentiation, or induction of nitrogen monoxide production.

[0035]

The food or beverage of the present invention is not particularly limited, and includes, for instance, processed agricultural and forest products, processed stock raising products, processed marine products and the like, including processed grain products such as processed wheat products, processed starch products, processed premix products, noodles, macaronis, bread, bean jam, buckwheat noodles, wheat-gluten bread, rice noodle, *fen-tiao*, and packed rice cake; processed fat and oil products such as plastic fat and oil, tempura oil, salad oil, mayonnaise, and dressing; processed soybean products such as tofu products, soybean paste, and fermented soybeans; processed meat products such as ham, bacon, pressed ham, and sausage; marine products such as frozen ground fish, boiled fish paste, tubular roll of boiled fish paste, cake of ground fish, deep-fried patty of fish paste, fish ball, sinew, fish meat ham and sausage, dried bonito, products of processed fish egg, marine cans, and preserved food boiled down in soy sauce (*tsukudani*); milk products such as raw material milk, cream, yogurt, butter, cheese, condensed milk, powder milk, and ice cream; processed vegetable and fruit products such as paste, jam, pickled vegetables, fruit beverages, vegetable beverages, and mixed beverages; confectionaries such as chocolates, biscuits, sweet bun, cake, rice cake snacks, and rice snacks; alcohol beverages such as *sake*, Chinese liquor, wine, whisky, Japanese distilled liquor (*shochu*), vodka, brandy, gin, ram, beer, refreshing alcoholic beverages, fruit liquor, and

liqueur; luxury drinks such as green tea, tea, oolong tea, coffee, refreshing beverages and lactic acid beverages; seasonings such as soy sauce, sauce, vinegar, and sweet rice wine; canned, binned or pouched foods such as rice topped cooked beef and vegetable, rice boiled together with meat and vegetables in a small pot, steamed rice with red beans, curry roux and rice, and other precooked foods; semi-dry or concentrated foods such as liver pastes and other spreads, soups for buckwheat noodles or wheat noodles, and concentrated soups; dry foods such as instant noodles, instant curry roux, instant coffee, powder juice, powder soup, instant soybean paste (*miso*) soup, precooked foods, precooked beverages, and precooked soup; frozen foods such as *sukiyaki*, pot-steamed hotchpotch, split and grilled eel, hamburger steak, *shao-mai*, dumpling stuffed with minced pork, various sticks, and fruit cocktails; solid foods; liquid foods (soups); spices; and the like.

[0036]

As the food or beverage of the present invention, there can be contained in, added to, diluted with the fucoidan and/or a degradation product thereof having action for regulation of cytokine production, immunopotentiation and induction of nitrogen monoxide production, and its shape is not particularly limited as long as an amount necessary for the fucoidan and/or a degradation product thereof to exhibit the physiological action is contained. The food or beverage includes, for instance, products shaped into tablets, granules, capsules or the like, which can be orally taken. Here, the fucoidan and a degradation product thereof having action for regulation of cytokine production are extremely useful production material for a food or beverage as a health food material having both physiological action and dietary fiber function.

[0037]

No case of death is found even when the fucoidan and/or a degradation product thereof having action for regulation of cytokine production, immunopotentiation, or induction of nitrogen monoxide production used in the present invention is orally administered to a rat in a single dose of 1 g/kg.

[0038]

[Examples]

The present invention will be more concretely described by means of the examples, without limiting the scope of the present invention thereto. Here, "%" in Examples means "% by weight."

[0039]

Reference Example 1

(1) *Kjellmaniella crassifolia* was sufficiently dried, and thereafter 20 kg of the dried product was powdered with a free mill (manufactured by Nara Kikai Seisakusho).

In 900 liters of tap water was dissolved 7.3 kg of calcium chloride dihydrate (manufactured by Nippon Soda Co., Ltd.), and 20 kg of the powdered product of *Kjellmaniella crassifolia* was then mixed therewith. The resulting mixture was heated for 40 minutes until the liquid temperature was raised from 12°C to 90°C by blowing steam. Thereafter, the mixture was kept at 90° to 95°C for 1 hour under stirring, and then cooled, to give 1100 liters of a cooled product.

Subsequently, the cooled product was subjected to solid-liquid separation with a solid-liquid separator (manufactured by West Farrier Separator, Model: CNA), to give about 900 liters of supernatant after solid-liquid separation.

The amount 360 liters of the supernatant after solid-liquid separation was

concentrated up to a volume of 20 liters with FE10-FC-FUS0382 (fraction molecular weight: 30000) manufactured by DAICEL CHEMICAL INDUSTRIES, LTD. Thereafter, the steps of adding 20 liters of tap water and again concentrating the resulting liquid mixture up to a volume of 20 liters were repeated 5 times, and the concentrate was subjected to a desalting treatment, to give 25 liters of an extract derived from *Kjellmaniella crassifolia*.

One liter of the extract was lyophilized, to give 13 g of a dried product of fucoidan derived from *Kjellmaniella crassifolia*.

[0040]

(2) Seven grams of the dried product of fucoidan described in item (1) of Reference Example 1 was dissolved in 700 ml of a 20 mM imidazole buffer (pH 8.0) containing 50 mM sodium chloride and 10% ethanol, and insoluble matters were removed by centrifugation. The supernatant after centrifugation was applied onto a DEAE-Cellulofine A-800 column (ϕ 11.4 cm x 48 cm) equilibrated with the same buffer, and then washed with the same buffer. The elution was carried out with a concentration gradient of from 50 mM to 1.95 M sodium chloride (250 ml per fraction). A total sugar content and an uronic acid content were determined by the phenol-sulfuric acid method and the carbazole-sulfuric acid method, to give Fractions 43 to 49, Fractions 50 to 55, and Fractions 56 to 67, in the order of elution. Next, these fractions were desalted by electrodialysis, and thereafter lyophilized, to give each of Fraction I (340 mg) from Fractions 43 to 49, Fraction II (870 mg) from Fractions 50 to 55, and Fraction III (2.64 g) from Fractions 56 to 67.

[0041]

Figure 1 shows an elution pattern of the fucoidan derived from

Kjellmaniella crassifolia on the DEAE-Cellulofine A-800 column. In Figure 1, the axis of ordinates is the absorbance at 530 nm as determined by the carbazole-sulfuric acid method (solid circles in the figure), the absorbance at 480 nm as determined by the phenol-sulfuric acid method (open circles in the figure), and the electric conductivity (mS/cm: open squares in the figure), and the axis of abscissas is the fraction number.

[0042]

Reference Example 2

(1) A 2-liter Erlenmeyer flask was charged with 600 ml of a culture medium comprising an artificial sea water (manufactured by Jamarin Laboratory), pH 8.2, containing 0.25% glucose, 1.0% peptone, and 0.05% yeast extract, and then sterilized (at 120°C for 20 minutes). *Alteromonas* sp. SN-1009 (FERM BP-5747) was inoculated into the culture medium, and cultured at 25°C for 26 hours, to give a seed culture medium. A 30-liter jar fermentor was charged with 20 liters of a culture medium comprising an artificial sea water, pH 8.0, containing 1.0% peptone, 0.02% yeast extract, 0.2% sulfated polysaccharide described in item (2) of Reference Example 2 described below, and 0.01% defoaming agent (manufactured by Shin-Etsu Chemical Co., Ltd., KM70), and sterilized at 120°C for 20 minutes. After cooling, 600 ml of the above-mentioned seed culture medium was inoculated, and cultured at 24°C for 24 hours under the conditions of 10 liters of aeration per minute and a stirring rate of 250 rpm. After termination of the culture, the culture medium was centrifuged, to give cells and culture supernatant. The culture supernatant obtained was concentrated with an ultrafilter equipped with holofiber having an excluding molecular weight of 10000, and the concentrate was then subjected to

salting out with an 85% saturated ammonium sulfate. Precipitates formed were harvested by centrifugation, and sufficiently dialyzed against a 20 mM Tris-HCl buffer (pH 8.2) containing an artificial sea water at a one-tenth concentration, to give 600 ml of a solution of an endo-sulfated polysaccharide-degrading enzyme, selectively acting on the sulfated polysaccharide.

[0043]

(2) Two kilograms of dried *Kjellmaniella crassifolia* was powdered with a cutter mill (manufactured by Masuko Sangyo) fitted with a screen having a diameter of 1 mm, and the resulting seaweed chips were suspended in 20 liters of 80% ethanol. The suspension was stirred at 25°C for 3 hours and filtered with a filter paper, and thereafter the residue was sufficiently washed. The residue obtained was suspended in 40 liters of a 20 mM sodium phosphate buffer, pH 6.5, which was heated to 95°C, the buffer containing 50 mM sodium chloride. The suspension was treated at 95°C for 2 hours with occasional stirring, to extract a sulfated polysaccharide.

The suspension of the extract was filtered, to give a filtrate. Thereafter, the filtration residue was washed with 3.5 liters of 100 mM sodium chloride, to give an additional filtrate.

Both filtrates were combined, and then the temperature was lowered to 30°C. After 3000 U of alginic acid lyase K (manufactured by Nagase Seikagaku Kogyo) was added to the resulting mixture, 4 liters of ethanol was added thereto. The resulting mixture was stirred at 25°C for 24 hours. Next, the mixture was centrifuged, and the resulting supernatant was concentrated up to a volume of 4 liters with an ultrafilter equipped with holofiber having an excluding molecular weight of 100000. Further, the ultrafiltration was continued with 100 mM

sodium chloride containing 10% ethanol until a colored substance was no longer filtered.

Precipitates formed in a non-filtrate solution were removed by centrifugation, and the temperature of the resulting supernatant was lowered to 5°C. The pH was adjusted to 2.0 with 0.5 N hydrochloric acid, and thereafter the formed precipitates such as a protein were removed by centrifugation. The pH of the resulting supernatant was rapidly adjusted to 8.0 with 1 N sodium hydroxide.

Next, an ultrafiltration was carried out with an ultrafilter equipped with holofiber having an excluding molecular weight of 100000, and the solvent was completely substituted with 20 mM sodium chloride, pH 8.0. Thereafter, the pH was again adjusted to 8.0, and the resulting mixture was centrifuged and then lyophilized, to give about 95 g of a sulfated polysaccharide.

[0044]

(3) Two kilograms of dried *Kjellmaniella crassifolia* was powdered with a cutter mill fitted with a screen having a diameter of 1 mm, and the resulting seaweed chips were suspended in 20 liters of 80% ethanol. The resulting suspension was stirred at 25°C for 3 hours, and filtered with a filter paper, and thereafter the residue was sufficiently washed. The residue obtained was suspended in 20 liters of a buffer (pH 8.2) containing 30 ml of a solution of the endo-sulfated polysaccharide-degrading enzyme prepared in item (1) of the above-mentioned Reference Example 2, 10% ethanol, 100 mM sodium chloride, 50 mM calcium chloride and 50 mM imidazole, and the resulting mixture was stirred at 25°C for 48 hours. This suspension was filtered with a stainless screen having a screen-opening diameter of 32 μm, and the residue was washed with 10% ethanol containing 50 mM calcium chloride. Further, the residue was

suspended in 10 liters of 10% ethanol containing 50 mM calcium chloride, and the suspension was stirred for 3 hours, and thereafter filtered with the stainless screen, and the residue was washed. Further, the residue was suspended under the same conditions, and the suspension was then stirred for 16 hours. The suspension was filtered with the stainless screen having a diameter of 32 μm, and the residue was washed.

The filtrate and the washings thus obtained were collected, and the combined mixture was subjected to ultrafiltration with an ultrafilter equipped with holofiber having an excluding molecular weight of 3000, thereby separating a filtered solution from a non-filtered solution.

This filtered solution was concentrated to a volume of about 3 liters with a rotary evaporator, and thereafter the concentrate was centrifuged, to give supernatant. The supernatant obtained was desalted with an electric dialyzer equipped with a membrane having an excluding molecular weight of 300. To the resulting solution was added calcium acetate so as to give a concentration of 0.1 M, and precipitates formed were removed by centrifugation. The resulting supernatant was applied onto a DEAE-Cellulofine column (amount of resin: 4 liters) previously equilibrated with 50 mM calcium acetate, and sufficiently washed with 50 mM calcium acetate and 50 mM sodium chloride. Thereafter, the elution was carried out with a gradient of from 50 mM to 800 mM sodium chloride. The amount collected at this time was 500 ml per fraction. The collected fraction was analyzed by cellulose acetate membrane electrophoresis [*Analytical Biochemistry*, **37**, 197-202 (1970)]. As a result, a sulfated saccharide which was eluted on a concentration of about 0.4 M sodium chloride (Proximity of Fraction No. 63) was homogeneous.

Then, a solution of Fraction No. 63 was first concentrated to a volume of 150 ml, and thereafter sodium chloride was added so as to give a concentration of 4 M. The resulting solution was applied onto a Phenyl-Cellulofine column (amount of resin: 200 ml) previously equilibrated with 4 M sodium chloride, and sufficiently washed with 4 M sodium chloride. Non-adsorptive sulfated saccharide fractions were collected, and desalted with an electrodialyzer equipped with a membrane having an excluding molecular weight of 300, to give 505 ml of a desalted solution.

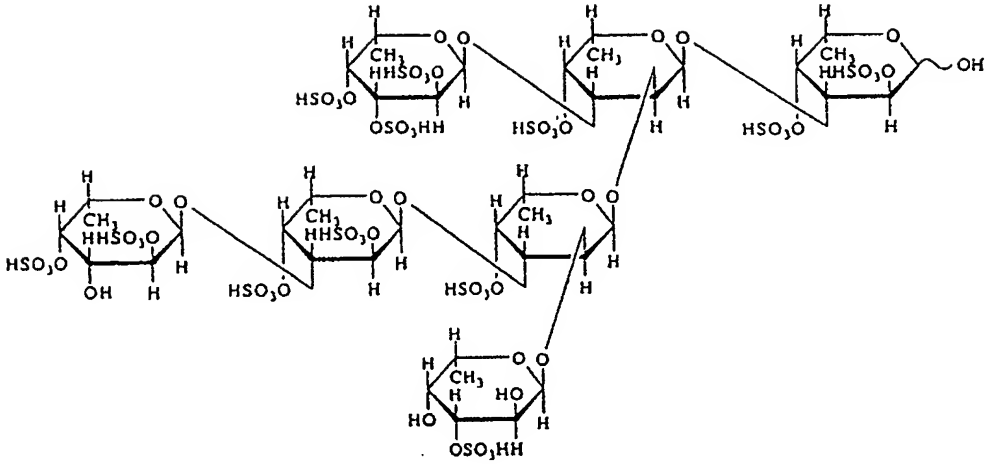
Forty milliliters of the desalted solution obtained was applied onto a Cellulofine GCL-90 column (4.1 cm x 87 cm) equilibrated with 0.2 M sodium chloride containing 10% ethanol, to perform gel filtration. The collection was performed at 9.2 ml per fraction.

All of the fractions were analyzed for a total sugar content by the phenol-sulfuric acid method [*Analytical Chemistry*, **28**, 350 (1956)].

As a result, since the sulfated saccharide formed a single peak, Fraction Nos. 63 to 70, which were fractions corresponding to a central part of the peak were collected. The combined fraction was desalted with an electrodialyzer equipped with a membrane having an excluding molecular weight of 300, and thereafter lyophilized, to give 112 mg of a dried product of the compound represented by the following formula V.

[0045]

[Ka 4]



[0046]

Reference Example 3

One kilogram of a dried product of a commercially available sporophyll of *Undaria pinnatifida* (Wakame Mekabu) was powdered with a cutter mill fitted with a screen having a hole diameter of 1 mm. Thereafter, the powdered sporophyll was suspended in 10 liters of 80% ethanol, and the suspension was stirred for 3 hours, and thereafter filtered with a filter paper, to give a residue. The residue was suspended in 20 liters of a 40 mM sodium phosphate buffer (pH 6.5) containing 50 mM sodium chloride, and treated at 95°C for 2 hours. The treated solution was cooled to 37°C, and thereafter ethanol was added thereto so as to give a concentration of 10%. 12000 U of a commercially available alginic acid lyase K (manufactured by Nagase Seikagaku Kogyo) was added thereto, and thereafter the mixture was stirred at room temperature for 24 hours. The resulting treated solution was centrifuged, and the resulting supernatant was concentrated to a volume of 2 liters with an ultrafilter equipped with holofiber having an excluding molecular weight of 100000. Thereafter,

precipitates formed were removed by centrifugation. The resulting supernatant was cooled to 5°C, and thereafter 0.5 N hydrochloric acid was added thereto to adjust the pH to 2.0. Subsequently, the resulting mixture was stirred for 30 minutes, and precipitates formed were removed by centrifugation. The pH of the resulting supernatant was adjusted to 8.0 with 0.5 N sodium hydroxide, and the solvent was substituted with 20 mM sodium chloride by ultrafiltration. The pH of the resulting solution was adjusted to 8.0, and thereafter the supernatant obtained by centrifugation was lyophilized, to give 90.5 g of fucoidan derived from sporophyll of *Undaria pinnatifida*.

[0047]

Reference Example 4

One kilogram of a dried product of powdered *Fucus vesiculosus* was suspended in 10 liters of 80% ethanol, and the suspension was stirred for 3 hours, and thereafter filtered with a filter paper, to give a residue. The residue was suspended in 30 liters of a 30 mM sodium phosphate buffer (pH 6.0) containing 100 mM sodium chloride, and treated at 95°C for 2 hours. After the treated solution was cooled to 37°C, 100 g of activated carbon was added, and the mixture was stirred for 30 minutes. After 3000 U of a commercially available alginic acid lyase K was added, ethanol was added so as to give a concentration of 10%, and the resulting mixture was stirred at room temperature for 24 hours. The resulting treated solution was centrifuged, and the supernatant was concentrated to a volume of 2 liters with an ultrafilter equipped with holofiber having an excluding molecular weight of 100000. Thereafter, precipitates formed were removed by centrifugation, and the resulting supernatant was ultrafiltered with an extract added, to remove a pigment. The non-filtered

solution obtained was cooled to 5°C, and thereafter 0.5 N hydrochloric acid was added thereto to adjust the pH to 2.0. Thereafter, the resulting solution was stirred for 30 minutes, and precipitates formed were removed by centrifugation. The pH of the supernatant was adjusted to 8.0 with 0.5 N sodium hydroxide, and the solvent was substituted with 20 mM sodium chloride by ultrafiltration. The pH of the resulting solution was adjusted to 8.0, and thereafter the supernatant obtained by centrifugation was lyophilized, to give 71 g of fucoidan derived from *Fucus vesiculosus*.

[0048]

Reference Example 5

Two grams of fucoidan derived from *Kjellmaniella crassifolia* prepared by the method described in item (1) of Reference Example 1 was dissolved in 100 ml of water, and the pH of the solution was adjusted to pH 3 with citric acid. Thereafter, the resulting mixture was treated at 100°C for 3 hours, to give a hydrolysate with the acid of the fucoidan. This hydrolysate was subjected to molecular weight fractionation by gel filtration on Cellulofine GCL-300 or Cellulofine GCL-25, to fractionate the hydrolysate into fractions exceeding MW 25000 (Fraction A); exceeding MW 10000 to MW 25000 (Fraction B); exceeding MW 5000 to 10000 (Fraction C); exceeding MW 2000 to 5000 (Fraction D); exceeding MW 500 to 2000 (Fraction E); and MW 500 or less (Fraction F). Further, each of these fractions and the hydrolysate were desalted, and then lyophilized, to give the hydrolysate and each fraction of the hydrolysate.

[0049]

Reference Example 6

Five kilograms of a commercially available, salt-preserved *Nemacystus*

decipiens was mixed with 20 liters of ethanol, and cut into thin pieces with scissors. The resulting mixture was allowed to stand overnight, and then filtered with a filter paper. The resulting residue was suspended in 12.5 liters of water, and treated at 95°C for 2 hours. After the treated solution was filtered with a filter paper, 2600 ml of a 2.5% cetyl pyridinium chloride solution containing 350 mM sodium chloride was added thereto, and the resulting mixture was allowed to stand for 3 days. The supernatant portion was discarded, the precipitate portion was centrifuged, and the resulting supernatant was also discarded. To the precipitates obtained was added 2.5 liters of 350 mM sodium chloride, and thereafter the mixture was homogenized with a homogenizer and centrifuged. The washing steps were repeated 3 times. Four-hundred milliliters of 400 mM sodium chloride was added to the precipitates obtained. Thereafter, the mixture was homogenized with a homogenizer, and ethanol was added thereto so as to give a concentration of 80%. The mixture was stirred for 30 minutes, and then filtered with a filter paper. Five hundred milliliters of 80% ethanol saturated with sodium chloride was added to the residue obtained, and thereafter the mixture was homogenized with a homogenizer. Ethanol saturated with sodium chloride was added to make the total amount 1 liter, and the mixture was stirred for 30 minutes and then filtered with a filter paper. The washing steps were repeated until the absorbance at 260 nm of the filtrate became 0 (zero) (usually 5 times). The residue obtained was dissolved in 1.5 liters of 2 M sodium chloride, and thereafter insoluble matters were removed by centrifugation. The resulting solution was allowed to flow through a column containing 100 ml of a DEAE-Cellulofine A-800 previously equilibrated with 2 M sodium chloride. Effluent fractions were concentrated to a volume of 2 liters

with an ultrafilter equipped with holofiber having an excluding molecular weight of 100000, and thereafter the solvent was substituted with 2 mM sodium chloride by an ultrafilter. The resulting solution was centrifuged, and the resulting supernatant was lyophilized, to give 22.9 g of fucoidan derived from *Nemacystus decipiens*.

[0050]

Reference Example 7

Five kilograms of sea cucumbers were dissected, and the organs were removed to collect somatic layers. Five-hundred milliliters of acetone was added per 200 g of the wet weight of the somatic layers, and the mixture was treated with a homogenizer. Thereafter, the homogenate was filtered, and the residue was washed with acetone until no more colored substances remained. This residue was dried with suction, to give 140 g of a dried product. To this dried product was added 2.8 liters of a 0.4 M saline, and the mixture was treated at 100°C for 1 hour. Thereafter, the mixture was filtered, and the resulting residue was sufficiently washed with a 0.4 M saline, to give 3.7 liters of an extract. To this extract was added 5% cetyl pyridinium chloride until no more precipitates were formed, and the formed precipitates were harvested by centrifugation. The precipitates were suspended in a 0.4 M saline, and then centrifuged again. One liter of a 4 M saline was added to the resulting precipitates, and the mixture was treated with a homogenizer. Thereafter, 4 liters of ethanol was added thereto with stirring, and the resulting mixture was stirred for 1 hour, and thereafter filtered, to give precipitates. The steps of suspending the precipitates in 80% ethanol and thereafter filtering the suspension were repeated until the absorbance at 260 nm of the supernatant became 0. The precipitates obtained were

suspended in 2 liters of a 2M saline, and insoluble matters were removed by centrifugation. The supernatant was ultrafiltered with an ultrafilter equipped with a membrane having an excluding molecular weight of 30000, and completely desalted. Thereafter, the resulting product was lyophilized, to give 3.7 g of fucoidan derived from sea cucumbers.

[0051]

Example 1

(1) RAW 264.7 cells (ATCC TIB 71) were suspended in a Dulbecco's modified Eagle's medium (manufactured by Bio Whittaker; 12-917 F) without phenol red containing 10% fetal bovine serum (manufactured by Gibco), 2 mM L-glutamine (manufactured by Life Technologies Oriental, 25030-149), so as to have a concentration of 6×10^5 /ml. The cell suspension was added to each well of a 48-well microtiter plate in a volume of 500 μl each. The cells were cultured at 37°C in the presence of 5% CO_2 gas for 12 hours. Each of aqueous solutions of the samples was added to each well, and the cells were cultured for additional 12 hours. Thereafter, the concentration of NO_2^- formed by the oxidation of NO in the medium was determined. Fraction I, Fraction II, and Fraction III of the fucoidan derived from *Kjellmaniella crassifolia* described in item (2) of Reference Example 1 and the compound represented by the formula (Ka 4) described in item (3) of Reference Example 2 (hereinafter referred to as 7-12S), which were used as the samples, were added so as to have a final concentration of 1, 10 or 100 μg/ml. Incidentally, as the positive control, a lipopolysaccharide (LPS, manufactured by Sigma) was added so as to have a final concentration of 1 μg/ml.

After the above culturing, 100 μl of a 4% Griess' reagent (manufactured

by Sigma, G4410) was added to 100 μl of the medium, and the resulting mixture was allowed to stand at room temperature for 15 minutes. Thereafter, the absorbance at 540 nm was determined. The NO_2^- concentration in the medium was calculated from a calibration curve previously drawn using $NaNO_2$ dissolved in the above medium at a known concentration. All determinations were carried out three times.

As a result, it was clarified that all of 7-12S, and Fraction I, Fraction II, and Fraction III of the fucoidan derived from *Kjellmaniella crassifolia* accelerated the induction of NO production, and had an immunopotentiating action. The results are shown in Figures 2 to 5. Concretely, Figure 2 is a diagram showing the NO_2^- concentration in the medium when culturing with adding 7-12S, Figure 3 is that with adding Fraction I, Figure 4 is that with adding Fraction II, and Figure 5 is that with adding Fraction III. Also, Figure 6 is a diagram showing the NO_2^- concentration in the medium when culturing with adding LPS as the control. In Figures 2 to 6, the axis of abscissas is the culture conditions, and the axis of ordinates is the NO_2^- concentration (μM).

It was clarified from these results that Fraction I, Fraction II, and Fraction III of the fucoidan derived from *Kjellmaniella crassifolia*, and 7-12S had an induction action for NO production and an immunopotentiating effect through the action.

Incidentally, the fucoidans described in each of Reference Examples also exhibited a similar induction action for NO production.

In addition, the amount of IFN-γ in the same culture medium as the culture medium used for the determination of the NO_2^- concentration was determined using ELISA Kit (Genzyme). However, in this system, no

enhancement of IFN-γ production by Fraction I, Fraction II, and Fraction III of the fucoidan derived from *Kjellmaniella crassifolia*, 7-12S, and other fucoidans described in each of Reference Examples was found.

[0052]

Example 2

(1) Induction Action for IFN-γ from Non-Stimulated Lymphocytes

An ICR mouse (female, 7-week old, body weight of about 25 g) was purchased from Japan SLC, and used for the experiment after pre-breeding the mouse for 1 week. The spleen was enucleated from the mouse, finely powdered, and suspended in RPMI-1640 medium (Gibco) containing 10% fetal bovine serum (Hiclone), to give a suspension of single cells. Adherent cells were removed by adhering them to a plastic petri dish, and non-adherent cells were used as spleen lymphocytes. Spleen lymphocytes were suspended in RPMI-1640 medium containing 10% fetal bovine serum, the concentration of which was adjusted to 2×10^6 cells/ml, and the cell suspension was added to a 96-well microtiter plate in a volume of 200 μl/well. Each of fucoidan, Fraction I, Fraction II, and Fraction III of the fucoidan derived from *Kjellmaniella crassifolia* and 7-12S described in Reference Examples, which had a given concentration, or 2 μg of concanavalin A (Con A; nakalaitesque) was added to each well except for the control group, and the cells were cultured in a 5% CO_2 gas incubator at 37°C for 2 days or 4 days. After culturing, the culture supernatant was recovered, and the amount of IFN-γ was determined using ELISA Kit (Genzyme).

As a result, no induction action for IFN-γ from non-stimulated lymphocytes was found for each of fucoidan, Fraction I, Fraction II and Fraction

III of the fucoidan derived from *Kjellmaniella crassifolia*, and 7-12S described in Reference Examples at a dose of 500 μg/ml or less. On the other hand, a strong induction for IFN-γ was found for the Con A-added cells.

[0053]

(2) Induction Action for IFN-γ Under Alloantigen Stimulation

A BALB/c mouse (female, 6-week old, body weight of about 20 g) and a C57BL/6 mouse (female, 6-week old, body weight of about 20 g) were purchased from Japan SLC, and used for the experiment after pre-breeding the mouse for 1 week. The spleens were enucleated from mice having different H-2 haplotypes (BALB/c: H-2^d, C57BL/6: H-2^b), and spleen lymphocytes were obtained by the method described above. The cell concentration of each cell suspension was adjusted to a concentration of 2×10^6 cells/ml, and added to a 96-well microtiter plate in a volume of 100 μl each. Each of fucoidan, Fraction I, Fraction II and Fraction III of the fucoidan derived from *Kjellmaniella crassifolia*, and 7-12S described in Reference Examples, which had a given concentration, or 2 μg of Con A was added to each well except for the control group. The cells were cultured in a 5% CO_2 gas incubator at 37°C for 4 days. After culturing, the culture supernatant was recovered, and the amount of IFN-γ was determined using the ELISA Kit.

[0054]

As a result, no induction action for IFN-γ on the lymphocytes in the state of alloantigen stimulation was found for each of fucoidan, Fraction I, Fraction II and Fraction III of the fucoidan derived from *Kjellmaniella crassifolia*, and 7-12S described in Reference Examples at a dose of 500 μg/ml or less. On the other hand, a strong induction of IFN-γ was found for the Con A-added cells.

[0055]

(3) Induction Action for IFN-γ on Sensitized Lymphocytes Under Antigen Stimulation

A C57BL/6 mouse (female, 6-week old, body weight of about 20 g) was purchased from Japan SLC, and used for the experiment after pre-breeding the mouse for 1 week. The mouse was immunized intraperitoneally by inoculating 1×10^6 cells of Meth-A murine sarcoma cells. Fourteen days after the inoculation of the tumor, the spleen was enucleated from the mouse, and spleen lymphocytes were obtained by the method described above. The cell concentration of the cell suspension was adjusted to a concentration of 2×10^6 cells/ml, and added to a 96-well microtiter plate in a volume of 100 μl each. For the preparation of stimulated cells, mitomycin C (KYOWA HAKKO KOGYO CO., LTD.) was added at a concentration of 50 μg/ml to Meth-A murine sarcoma cells which were suspended in RPMI-1640 medium, the concentration of which was adjusted to 2×10^6 cells/ml. The mixture was treated at 37°C for 30 minutes, and washed twice. Thereafter, the cells were suspended in RPMI-1640 medium containing 10% fetal bovine serum, the concentration of which was adjusted to 2×10^6 cells/ml. The prepared stimulated cells were overlaid in an amount of 100 μl/well onto each well of the plate containing spleen lymphocytes, and cultured in a 5% CO_2 gas incubator at 37°C for 4 days. Those were added so that the fucoidan derived from *Kjellmaniella crassifolia* had a concentration of 1 to 100 μg/ml; Fraction I, Fraction II and Fraction III of the fucoidan derived from *Kjellmaniella crassifolia* and 7-12S each had a concentration of 10 to 500 μg/ml, and the cells were cultured. As the control, 2 μg of Con A was added, and the cells were cultured. After culturing, the

culture supernatant was recovered, and the amount of IFN-γ was determined using the ELISA Kit. The amount of IL-12 was determined for the same culture supernatant using ELISA Kit (ENDOGEN).

[0056]

The results are shown in Figures 7 and 8. Concretely, Figure 7 is a diagram showing the induction action for IFN-γ production by the fucoidan and the degradation product thereof. In the figure, the axis of ordinates is the amount of IFN-γ production (pg/ml), and the axis of abscissas is each sample and added amount (μg/ml).

Also, Figure 8 is a diagram showing the induction action for IL-12 production by the fucoidan and the degradation product thereof. In the figure, the axis of ordinates is the amount of IFN-γ production (pg/ml), and the axis of abscissa is each sample and added amount (μg/ml).

As shown in Figures 7 and 8, there are exhibited enhancing action for IFN-γ production and IL-12 production on sensitized lymphocytes under antigen stimulation in a dose-dependent manner at a dose of 1 to 100 μg/ml for the fucoidan derived from *Kjellmaniella crassifolia*, and at a dose of 10 to 500 μg/ml for Fraction I, Fraction II, Fraction III and 7-12S. In addition, fucoidans described in each of Reference Examples also exhibited similar action.

[0057]

Example 3

A C57BL/6 mouse (female, 7-week old, body weight of about 20 g) was purchased from Japan SLC, and used for the experiment after pre-breeding the mouse for 1 week. Fifty micromoles of the LCMV (lymphocytic choriomeningitis virus)-derived peptide of SEQ ID NO: 1 shown in Sequence

Listing which binds to a murine major histocompatibility antigen class-I [hereinafter referred to as LCMV; European Journal of Immunology, **28(10)**, 3301-3311 (1998)] and 100 μmol of the HBV (Hepatitis B virus)-derived peptide of SEQ ID NO: 2 shown in Sequence Listing which binds to a murine major histocompatibility antigen class-II [hereinafter referred to as HBV; Journal of Virology, **69(5)**, 2776-2785 (1995)] as a helper peptide were mixed with complete Freund's adjuvant to give an emulsion, and the mouse was immunized subcutaneously with the emulsion at the neck of the tail of the mouse. Ten days after the immunization, the spleen was enucleated from the mouse, finely powdered, and suspended in RPMI-1640 medium (Bio Whittaker) containing 10% fetal bovine serum (Hiclone), to give a suspension of single cells. Spleen lymphocytes were suspended in RPMI-1640 medium containing 10% fetal bovine serum, the concentration of which was adjusted to 3×10^6 cells/ml, and added in a volume of 10 ml to a T25 flask (Iwaki), and the LCMV peptide was added so as to have a concentration of 100 ng/ml. Two identical flasks were furnished: One flask to which the medium alone was added, and the other flask to which the fucoidan derived from *Kjellmaniella crassifolia* was added so as to have a concentration of 10 μg/ml. The cells were cultured at 37°C in the presence of 5% CO_2.

Ten days after the initiation of the culturing, the cells were harvested, diluted with RPMI-1640 medium containing 10% fetal bovine serum so as to have a given concentration, and added to each well of a 96-well round-bottomed microplate in a volume of 100 μl each.

[0058]

As the cytotoxic activity of the cytotoxic T cells, the amount of γ-ray

liberated in the culture supernatant was determined using ^{51}Cr-labeled EL4 thymoma cells as target cells. Concretely, 1850 kBq of Chromium-51 Radionuclide (New England Nuclear) was added to EL4 cells, and the cells were cultured at 37°C for 1 hour and washed thrice with RPMI-1640 medium by a centrifugation step. The cells were suspended in RPMI-1640 medium containing 10% fetal bovine serum, the concentration of which was adjusted to 1×10^5 cells/ml. The suspension was added to each well of the above-mentioned 96-well microplate. The LCMV peptide was also added at the same time so as to have a concentration of 10 μg/ml. The cells were cultured at 37°C in the presence of 5% CO_2 for 5 hours. One-hundred microliters of the supernatant was taken, and thereafter the amount of γ-ray liberated in the supernatant was determined with a gamma ray scintillation counter. The cytotoxic activity was calculated as follows.

[0059]

[Su 1]

$$\text{Cytotoxic Activity (\%)} = \frac{(\text{Experimental Value} - \text{Control Value})}{(\text{Total Radioactivity Value} - \text{Control Value})} \times 100$$

[0060]

The results are shown in Figure 9. Concretely, Figure 9 is a diagram showing enhancing action of cytotoxic T cells in murine spleen lymphocytes by the fucoidan derived from *Kjellmaniella crassifolia*. The axis of ordinates is cytotoxic activity (%), and the axis of abscissas is the ratio of the effector cells of the control and the effector cells which were obtained by culturing the cells with adding the fucoidan derived from *Kjellmaniella crassifolia* described in

Reference Example 1 so as to have a concentration of 10 μg/ml, to target cells. The bar graph shows the average value for 5 mice per each group and the standard error.

As shown in Figure 9, the LCMV peptide-immunized, cultured murine splenocytes induced a cytotoxic activity to EL4 cells (control) which was labeled with the same peptide as above. In the cells cultured with adding the fucoidan so as to have a concentration of 10 μg/ml, the cytotoxic activity to EL4 cells was potentiated. The fucoidan-added group exhibited higher cytotoxic activity in spite of a lower ratio of the effector cells to target cells.

The EL4 cells are resistant to natural killer activity, and the activity of the cytotoxic T cells specific to the antigen was enhanced by the fucoidan. This is considered to be caused by the fact that precursor cells of the cytotoxic T cells induced by immunization with an antigenic peptide were matured to show cytotoxic activity when the precursor cells were cultured with adding the antigenic peptide, and the fucoidan stimulates the antigen presenting cells at this time, so that the activity of the cytotoxic T cells was enhanced via production of a cytokine such as IL-12.

In addition, each of other fucoidans, Fraction I, Fraction II, Fraction III, and 7-12S described in Reference Examples also exhibited similar activity. The enhancement of activity of the cytotoxic T cells by the fucoidan can not only be applied to the treatment of lymphatic choriomeningitis virus but also to the treatment of other viral infections and malignant tumors.

[0061]

[Effects of the Invention]

According to the present invention, there is provided a medicament

effective for a disease requiring regulation of cytokine production, a disease requiring immunopotentiation and a disease requiring nitrogen monoxide production, wherein the medicament comprises as an effective ingredient a substance exhibiting an action for regulation of cytokine production, an action for immunopotentiation and an action for induction of nitrogen monoxide production. The medicament has an regulatory activity for production of an interleukin, an interferon, or the like in a living body, so that the medicament is useful as a therapeutic agent or prophylactic agent for a disease requiring regulation of cytokine production such as cancers and immunological diseases.

In addition, the medicament is especially useful as an agent for induction of IFN-γ production, an agent for induction of IL-2 production, an agent for enhancement of cytotoxic T cells, and an agent for induction of NO production for diseases requiring applications of these agents.

[0062]

Further, a foodstuff can be produced by using the fucoidan and/or degradation product thereof, the foodstuff having an action for regulation of cytokine production, an action for immunopotentiation and an action for induction of nitrogen oxide production. By an intake of the fucoidan and/or degradation product thereof on a daily basis as foodstuff, there can be accomplished amelioration of symptoms of a disease requiring regulation of cytokine production, a disease requiring immunopotentiation, and a disease requiring nitrogen monoxide production such as arteriosclerosis, or the like.

Therefore, the functional foodstuff comprising as an effective ingredient the fucoidan and/or degradation product thereof is a functional foodstuff useful for maintaining homeostasis of a living body due to the physiological action of

the effective ingredient.

In addition, there is provided an agent for regulation of cytokine production, and the agent is useful for function studies on cytokine and screening for a medicament for cytokine-associated diseases.

[0063]

[Sequence Listing]

SEQUENCE LISTING

<110> Takara Shuzo Co., Ltd.

<120> Pharmaceutical Agents

<130> T-1341

<160> 2

<210> 1

<211> 9

<212> PRT

<213> Lymphocytic choriomeningitis virus

<400> 1

Lys Ala Val Tyr Asn Phe Ala Thr Met

1 5

<210> 2

<211> 13

<212> PRT

<213> Hepatitis B virus

<400> 2

Thr Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu

1 5 10

[Brief Description of the Drawings]

[Figure 1]

A graph showing an elution pattern of the fucoidan derived from *Kjellmaniella crassifolia* on DEAE-Cellulofine A-800 column.

[Figure 2]

A graph showing the NO_2^- concentration in the medium when cells are cultured with adding 7-12S.

[Figure 3]

A graph showing the NO_2^- concentration in the medium when cells are cultured with adding Fraction I.

[Figure 4]

A graph showing the NO_2^- concentration in the medium when cells are cultured with adding Fraction II.

[Figure 5]

A graph showing the NO_2^- concentration in the medium when cells are cultured with adding Fraction III.

[Figure 6]

A graph showing the NO_2^- concentration in the medium when cells are cultured with adding LPS as the control.

[Figure 7]

A graph showing the action for induction of IFN-γ production by the fucoidan and a degradation product thereof.

[Figure 8]

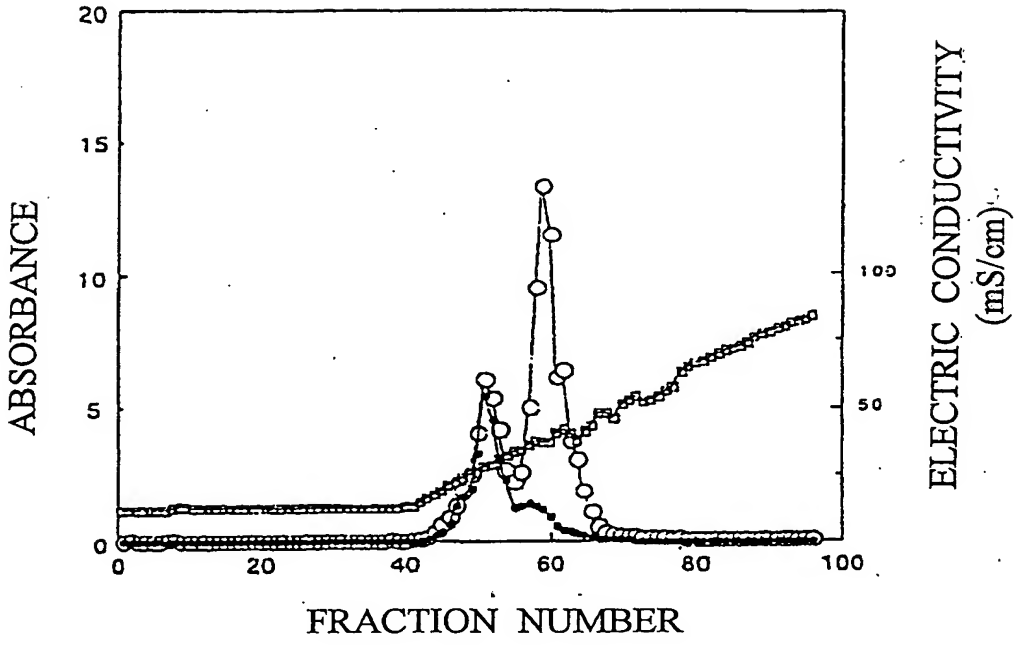
A graph showing the action for induction of IL-12 production by the fucoidan and a degradation product thereof.

[Figure 9]

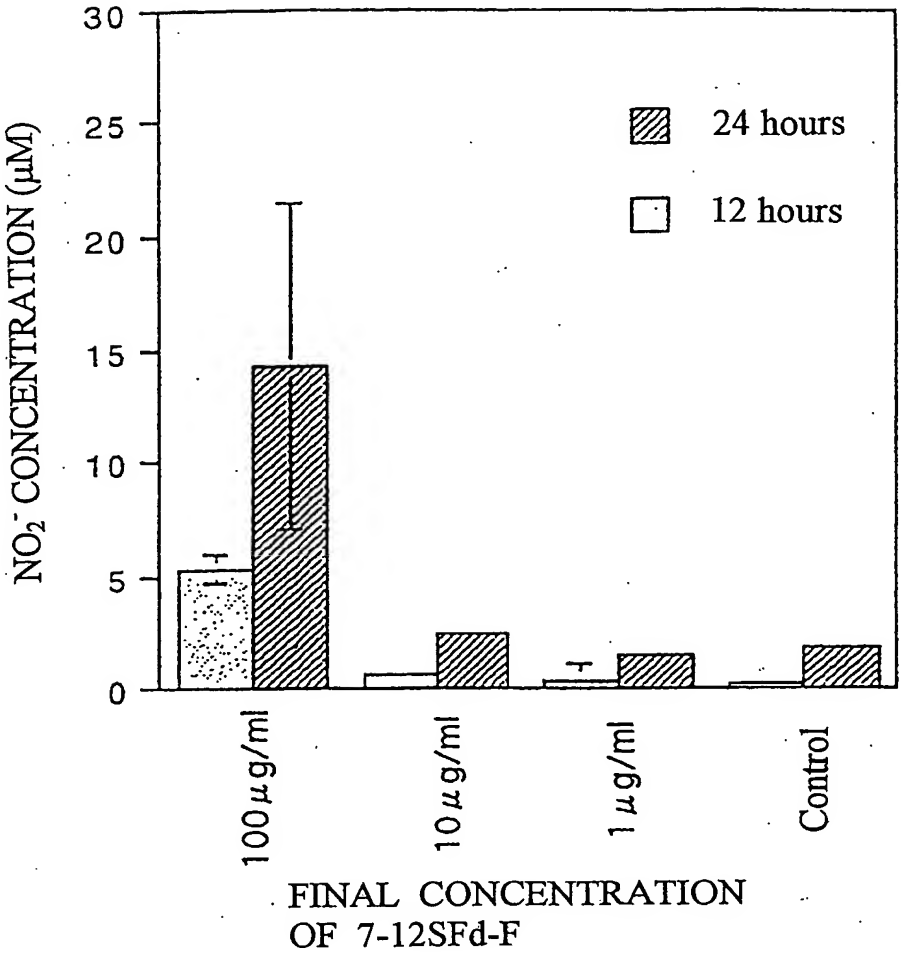
A graph showing the enhancing action for cytotoxic T cell in murine spleen lymphocytes by the fucoidan derived from *Kjellmaniella crassifolia*.

[Document]

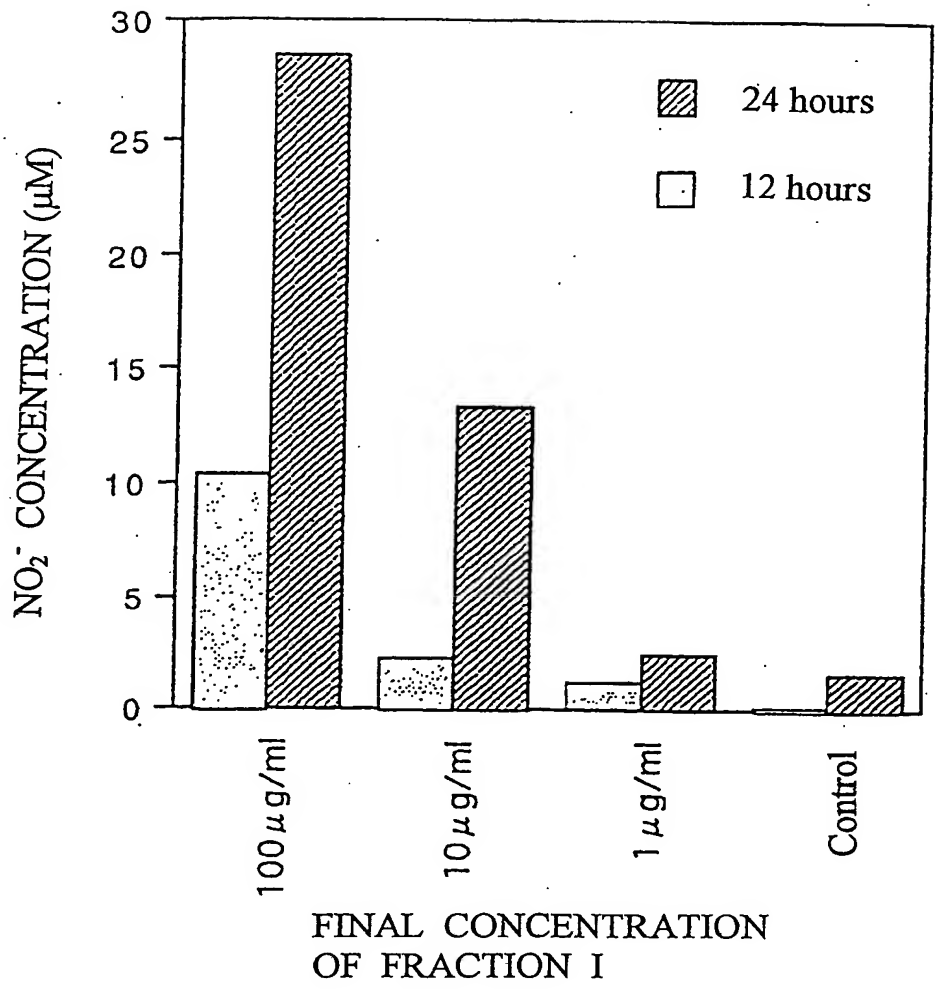
[Figure 1]



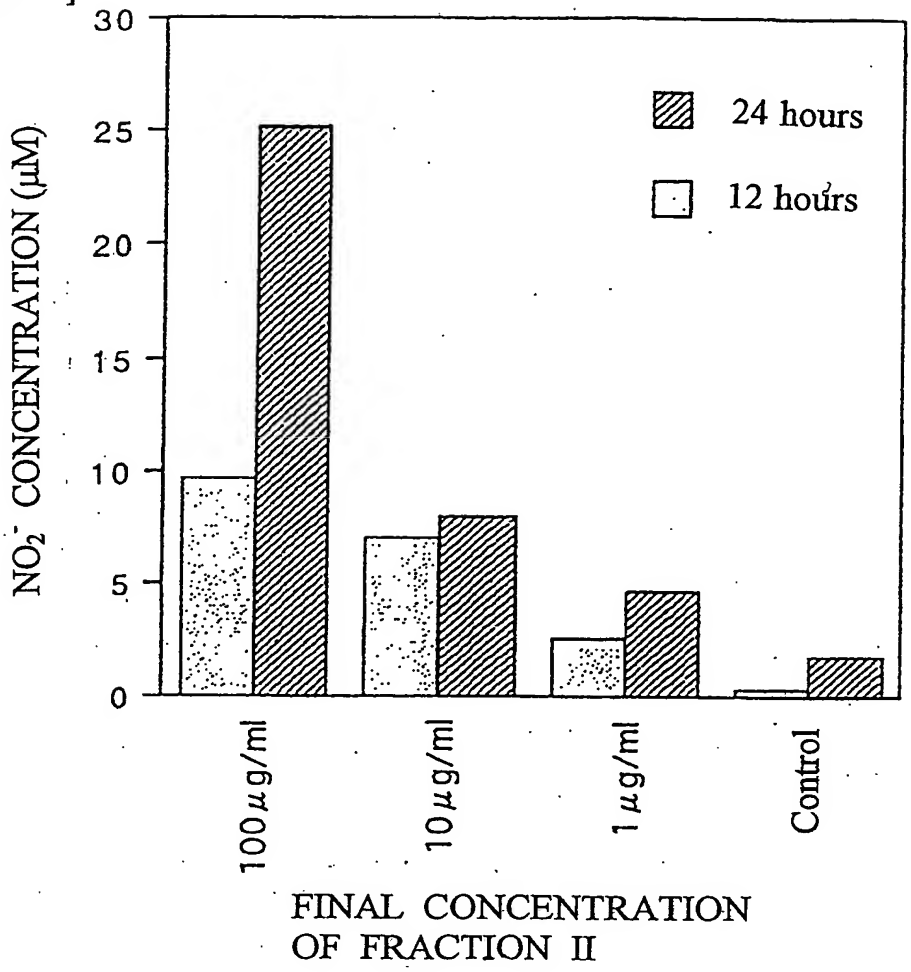
[Figure 2]



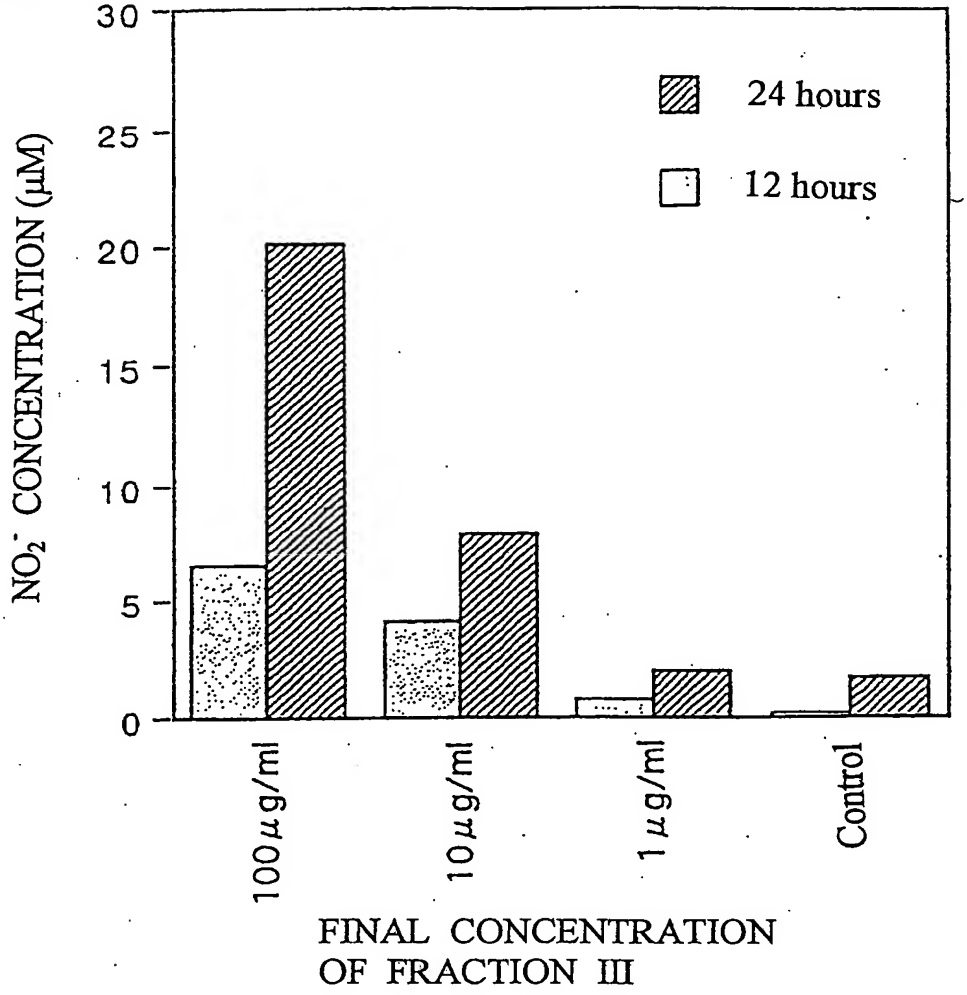
[Figure 3]



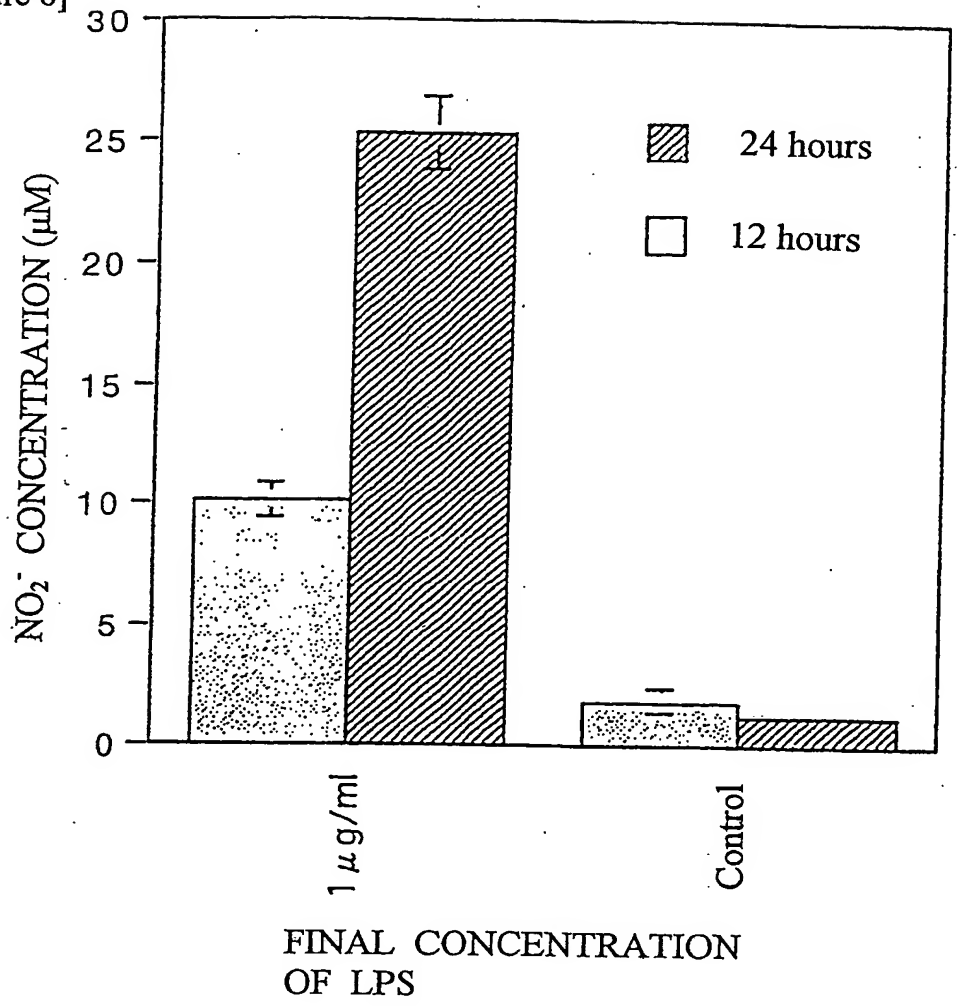
[Figure 4]



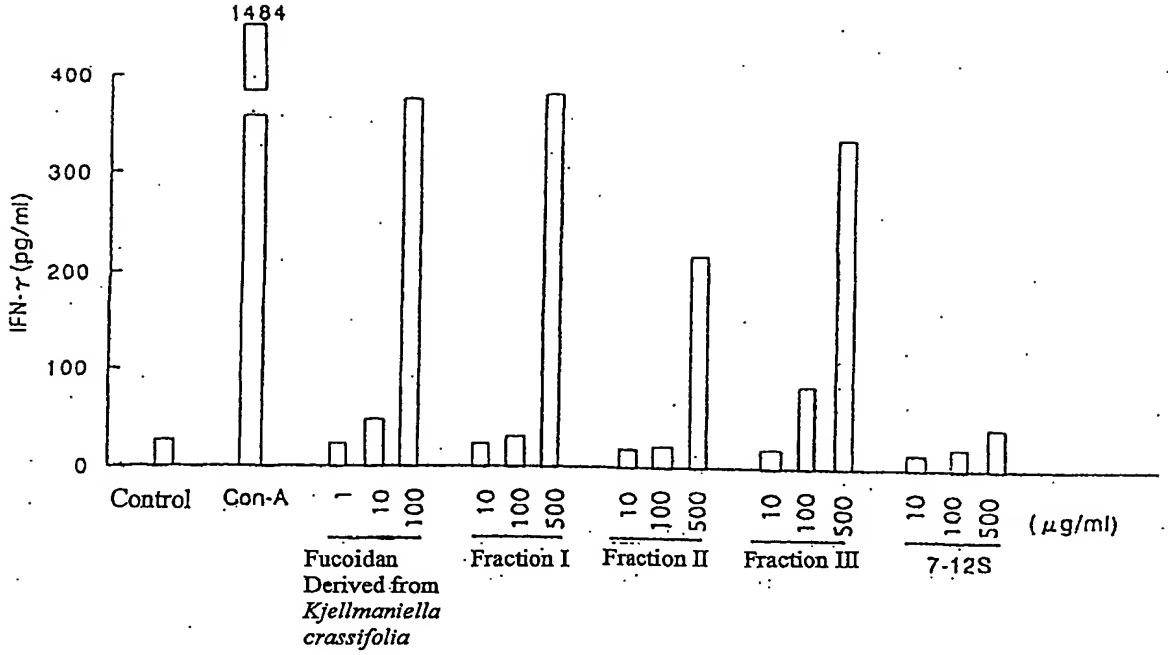
[Figure 5]



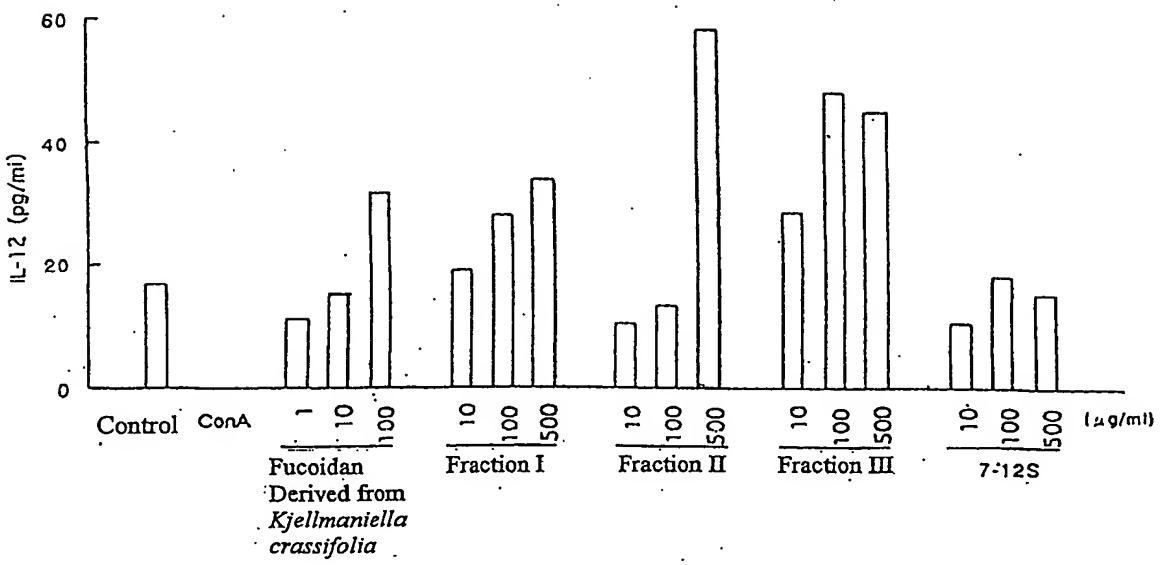
[Figure 6]



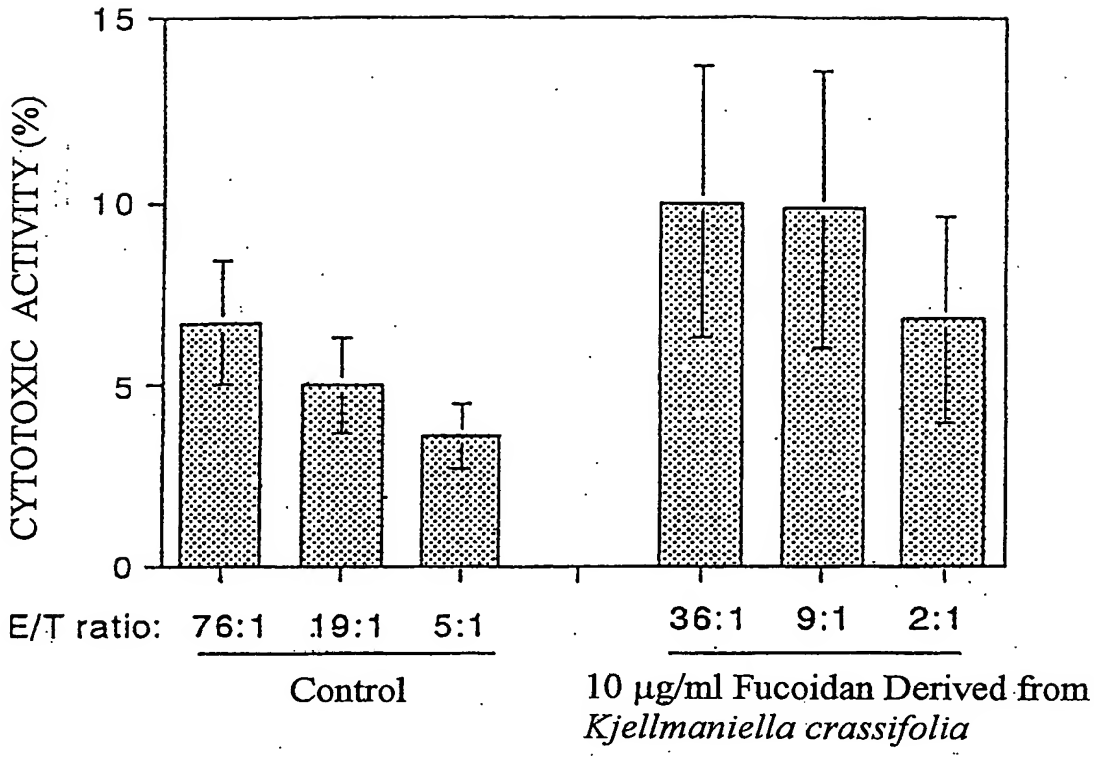
[Figure 7]



[Figure 8]



[Figure 9]



[Document] Abstract

[Abstract]

[Problems]

To provide a medicament, food or beverage utilizing action for regulation of cytokine production or the like of a fucoidan.

[Solving Means]

A therapeutic agent or prophylactic agent for a disease requiring regulation of cytokine production, a disease requiring immunopotentiation or a disease requiring nitrogen monoxide production, characterized in that the therapeutic agent or prophylactic agent comprises as an effective ingredient a fucoidan and/or a degradation product thereof; and a food or beverage for regulation of cytokine production, a food or beverage for immunopotentiation, or a food or beverage for induction of nitrogen monoxide production, wherein a fucoidan or a degradation product thereof is contained in, added to, and/or diluted in the food or beverage.

[Selected Drawings] None